^{125}I-labelled Hp(1-1)-Hb complexes although a lower rate of uptake was observed in comparison with the ^{125}I-labelled Hp(2-2)-Hb complexes (data not shown). The SU-DHL cell line expresses, in addition to the most abundant CD163 variant, also two less abundant variants Law, S. K. *et al.* A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *Eur. J. Immunol.* **23**, 2320-2325 (1993) with different cytoplasmic tails.

Example 5

Methods of detection and measuring of soluble CD163 (sHbSR) in plasma and serum

Soluble CD163 has been detected in plasma in normal human subjects by ELISA and Western blotting. The western blot shows a protein of identical electrophoretic mobility as full length HbSR/CD163 indicating that the protein in plasma either represents the full length protein or only a slightly truncated protein. Because the protein is soluble in plasma we designate it soluble CD163 (sHbSR)

The following Sandwich-ELISA-type assay for measuring the concentration of sHbSR has been developed:

-Polyclonal antibody (Rabbit-antiCD163, produced by DAKO for S.K.Moestrup) is coated onto micro-titer wells (concentration in buffer 4 mg/l). Plates are kept at 4°C until use.

-The wells are washed 3 times in phosphate-buffered saline (PBS), and 100 microliter (µl) of each sample (e.g. plasma or serum, diluted 50 times in PBS with albumin) is subsequently added to the wells. The samples incubate for 1 hour at 22°C with agitation.

-The wells are washed again 3 times in PBS, and 100 µl of monoclonal antiCD163 (GHi/6, produced by PharMingen, diluted 500 times in PBS with albumin) is added to each well. The antibody incubates for 1 hour at 22°C with agitation.

-The wells are washed again 3 times in PBS, and 100 µl of polyclonal, peroxidase-labeled antibody (Goat-antirabbit (P447) produced by DAKO, diluted 8000 times in PBS with albumin) is added to each well. The antibody incubates for 1 hour at 22°C with agitation.

-The wells are washed again 3 times in PBS, and 100 µl of a substrate-solution (OPD, orthophenyldiamine, with H_2O_2 added) is added to each well, and the colour-development is subsequently stopped after 15-30 min by addition of 50 µl of 1 M H_2SO_4.

-The intensity of the colour is proportional to the concentration of sHbSR in the sample, and is measured in a micro-plate reader at a wavelength of 495 nm (using 620 nm as a reference). Standards with known concentrations of sHbSR are analysed in the same way on the

same plate, and a standard curve can be produced. The colour-intensity of the sample, therefore can be transformed into concentration by comparing with the standard curve (fig. 8).

Assay-characteristics

Assay precision: Coefficient of variation = 2-4 % in the measuring range (intraserial)

Detection limit (the minimum measurable concentration): approximately 0.2 µg/l

Bias: no matrix effect has been observed in plasma samples of different dilution

Specificity: In western-blots (of serum after affinity-purification with polyclonal anti-CD163, and subsequent blotting with monoclonal antiCD163) one single band is observed, with a molecular size corresponding to soluble HbSR. For Western blotting, sHbSR in 100 µl plasma is initially captured by a polyclonal anti-human HbSR/CD163 antibody linked to Sepharose. The beads are washed and subjected to traditional non-reducing SDS-gelelectrophoresis and western blotting with a monoclonal anti-human HbSR/CD163 antibody. The capturing reagent and detecting reagent may be modified as in the ELISA assay described above.

Concentration of sCD163 in blood donors and patients

The mean concentration of sHbSR in plasma from 31 blood donors was 265 µg/l.

The concentration in 31 paired serum samples was not different 264 µg/l), indicating that both sample types can by used in the assay.

In preliminary experiments, randomly assayed samples from patients from a hematological department, have shown values ranging from the normal values found in blood donors to values 5-10 times higher.

Example 6

Uptake in HbSR expressing cells of a heterogeneous moiety covalently linked to Hb-Hp

The uptake was tested on transfected CHO-cells recombinantly expressing wt HbSR (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and K.,M.S.K. (2001) Identification of the hemoglobin scavenger receptor, *Nature 409*, 198-201), CHO-cells expressing the human receptor cubilin (Kristiansen,M., Kozyraki,R., Jacobsen,C., Nexo,E., Verroust,P.J., and Moestrup,S.K. (1999) Molecular dissection of the intrinsic factor-vitamin B12 receptor, cubilin, discloses regions important for membrane association and ligand binding, *J. Biol. Chem. 274*, 20540-20544)

was used as control. Cells were grown on chamber slides (Lab Tek permanox slide Nalge Nunc International) at 37°C and 5 % CO_2 for 20 hours. Each well was incubated for 1 hour at 37°C and 5 % CO_2 with 300 µL of CHO-media (hyQ-CCM5, HyClone (Utah, USA)) added Alexa Flour 488 labeled Hp(2-2)-Hb (labeled using the Alexa Flour 488 Protein Labeling Kit (Molecular Probes, Oregon)) to a final concentration of 0.1 µM. The wells were washed twice with PBS pH 7.4 and incubated for 30 min. at room temperature with Ellis buffer (PBS pH 7.4 and 4 % formaldehyde). Washed three times with PBS pH 7.4, 0.05 % Triton X-100 and incubated for 1 hour at room temperature with PBS pH 7.4, 0.05 % Triton X-100 added rabbit derived polyclonal antibody recognizing either HbSR or cubilin (control cells), with a final concentration of antibody of 10 µg/ml. Wells were washed trice in PBS pH 7.4, 0.05 % Triton X-100 and incubated for 1 hour at room temperature with PBS pH 7.4, 0.05 % Triton X-100 added Alexa Flour 594-labeled goat anti-rabbit IgG (Molecular Probes, Oregon) at a concentration of 5 µg/ml. Finally the wells were washed three times with PBS pH 7.4, 0.05 % Triton X-100 and overlaid with a cover plate and the fluorescence studied in the confocal microscope, see figure 9.

As can be seen both receptors react positively with their respectively antibody; red color. Only the cells expressing HbSR also take up Alexa Flour 488 labeled Hp-Hb; green color, whereas the mock cells, expressing cubilin, do not take up Hp-Hb. The distinct coloring pattern of Alexa Flour 488 in CHO cells expressing HbSR indicates that the complex is degraded in the lysomes of the cell. This result shows that a heterogeneous moiety can be coupled to Hp-Hb and selectively taken up by cells expressing HbSR, which in vivo natively will be macrophages.

Example 7

Localization of the Hp-Hb binding region of HbSR

Expression of recombinant soluble HbSR

A recombinant soluble HbSR derivative consisting of the extracellular domain (SRCR 1-9) without transmembrane segment and cytoplasmic tail was expressed in Chinese Hamster Ovary (CHO) cells stably tranfected with a HbSR cDNA fragment encoding amino acid 1-1045 of human HbSR. The cDNA plasmid was generated by the following procedure: Initially, a cDNA fragment corresponding to the bases 3045 to 3135 with the addition of a stop codon and a Not I site was created by PCR using the primers: 5´caa gga aga cgc tgc agt gaa ttg c3´and 5´tca gcg gcc gcc tag gat gac tga cgg gat gag cg3´ with full-length HbSR cDNA (Kristiansen,M., Graversen,J.H., Jacobsen,C., Sonne,O., Hoffman,H., Law,A.S.K., and